The Testis and Reproduction in Male *Necturus*, with Emphasis on *N. lewisi* (Brimley)

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ABSTRACT.— Although it has long been recognized that estrogens are produced by the testis of many vertebrate species the intratesticular site of aromatization is still controversial. Both interstitial Leydig and intertubular Sertoli cells have been implicated as the source of testicular estrogen. The mammalian testis is histologically complex with seminiferous tubules uniformly distributed amongst the interstitial tissue throughout the testis, which makes it difficult to localize steroidogenic enzymes within the testis. Urodele amphibians, however, at the close of the breeding season develop a specialized region of the testis called the glandular tissue that is essentially composed of Leydig cells and is formed by hypertrophy of the interlobular Leydig cells following spermiation of the seminiferous lobules. This glandular tissue in *Necturus* testis can be visualized with a dissecting microscope and separated from the seminiferous lobules, an anatomical arrangement that provides an opportunity to investigate the intratesticular location of steroidogenic enzymes. In a previous study it was shown that aromatase was localized to the glandular tissue in *Necturus maculosus* testis. Thus in *N. maculosus* Leydig cells are responsible for the production of testicular estrogens. The testis of *Necturus* also undergoes a longitudinal wave of spermatogenesis. Due to this topographical arrangement it is possible by dissection of the testis to obtain regions with Leydig cells at different stages of differentiation. This was carried out in *N. lewisi* towards the end of the breeding season at which time the testis was divided into cephalic and caudal regions. The cephalic region contained seminiferous lobules filled with germ cells and undifferentiated interlobular Leydig cells. The caudal region contained lobules that had undergone spermiation and the Leydig cells had hypertrophied to form the glandular tissue. Microsomes were prepared from these two regions and key enzymes for estrogen synthesis, 17α-hydroxylase and aromatase were measured. Cytochrome P-450, the catalytic component of these enzymes, was also measured in each

region. The results showed that levels of 17α-hydroxylase and aromatase as well as P-450 concentration increased as the glandular tissue developed. As yet the functional significance of estrogen production by *Necturus* testis has not been investigated.

INTRODUCTION

Urodele amphibians of the genus *Necturus* are widely distributed throughout the eastern and middle regions of North America, where they are extremely abundant in rivers tributary to the Great Lakes and to inland streams and small lakes. The ability to survive in waters of such diverse characteristics apparently accounts for their wide distribution. *Necturus*, one of the largest of salamanders, is primarily nocturnal, although in very muddy or reedy habitats it may be more or less active during the day, and is preeminently aquatic. These salamanders are also usually active throughout the whole year and do not, it seems, undergo periods of true hibernation.

The sexes of *Necturus* are similar in both form and coloration. Following a primitive courtship behavior the animals mate, depending on their regional distribution, throughout the summer and fall. Fertilization is internal by means of spermatophores produced by the males. The male vent is larger than that of the female, becomes inflamed during the breeding season, and is capable of eversion to expose two papillae that possibly aid in the deposition of spermatophores into the cloaca of the female. Cloacal glands occur in the male and are presumably involved in the formation of spermatophores. The spermatozoa are stored over the winter months in spermathecae of the females, which then undergo a short spawning season in the following spring and deposit eggs in rudimentary nests that can be guarded by either parent.

MORPHOLOGY OF NECTURUS *TESTIS*

As in all amphibia, the paired elongate testes of *Necturus* are abdominal in position, bordering the kidneys and attached to the dorsal body wall by a mesorchium (Fig. 1). Within this mesenteric membrane lies the vascular system of the testis and vasa efferentia, which convey the spermatozoa to the Wolffian ducts — long, coiled structures in which spermatozoa are stored prior to encapsulation within the spermatophores.

The structural unit of *Necturus* testes is the cyst which contains the germ cells and associated somatic cells, analogous to Sertoli cells found in amniote testes. These units are enclosed and contained in larger structures, the seminiferous lobules. Surrounding the lobules is the interlobular tissue in which is found the Leydig cells. The organization of the testis consists of seminiferous lobules radiating outward to the periphery

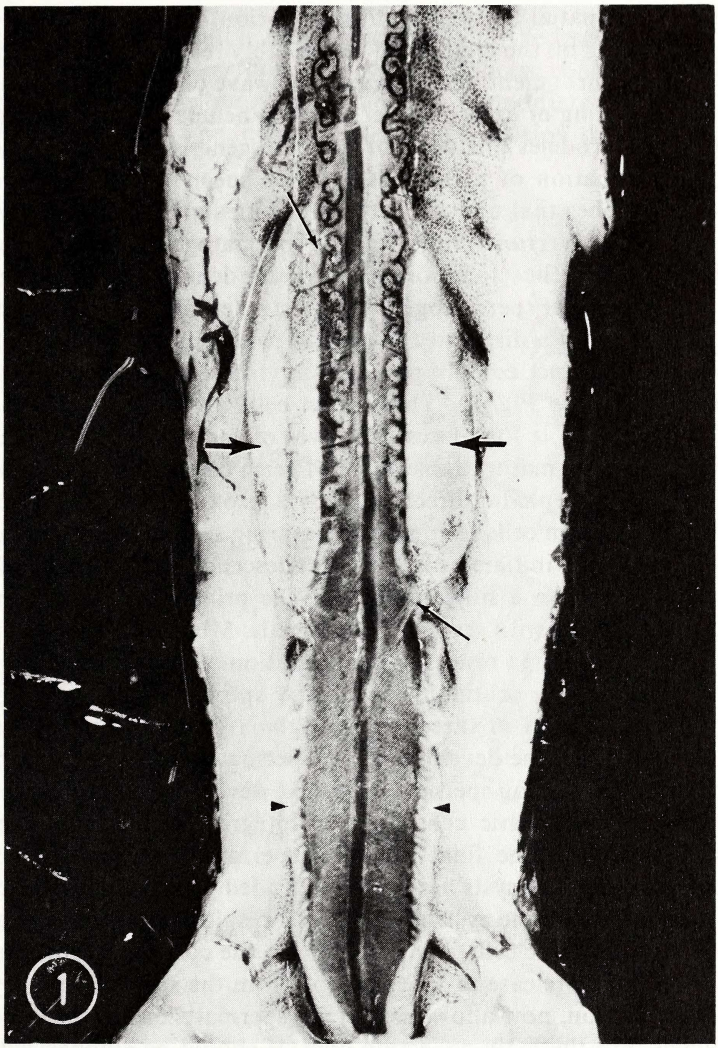

Fig. 1. Gross dissection of *Necturus maculosus* displaying testes (large arrows) attached to the dorsal body wall by a mesorchium (small arrows) through which run the vasa efferentia that empty into the Wolffian duct (arrowheads). From Pudney et al. (1983). X 1.5.

of the testis from a main, central, longitudinal collecting duct to which they are joined by short tubes devoid of germ cells (see Fig. 3). The main duct, in turn, is connected to the vasa efferentia, thus allowing egress of spermatozoa from the testis.

In *Necturus,* as in most urodeles, there is during the breeding cycle a caudocephalic wave of spermatogenesis along the length of the testis,

resulting in a spatial and temporal segregation of differentiating germ cells. Depending on the region and presumably regulated by the temperature of the environment, for *Necturus* this wave of spermatogenesis can begin in the spring or early summer and is concluded by late summer or fall. In many urodeles this wave of spermatogenesis is slow. Due to the tardy differentiation of germ cells in these species the testis becomes divided into lobes that contain germinal elements at different stages of development. In *Necturus*, however, the spermatogenic wave is comparatively rapid and thus lobation of the testis does not occur. Furthermore, the process of spermatogenesis is restricted to those regions of the seminiferous lobules distal to the central collecting duct, while lobules proximal to this duct contain groups of undifferentiated germ cells, the spermatogonia (see Fig. 2). These germ cells are in a "resting stage," providing a reservoir for successive waves of spermatogenesis. Thus, in *N. lewisi* there is a maturational wave of germ cells occurring longitudinally in a caudocephalic direction, plus a proximal to distal cycle of differentiating germ cells to form the maturing segments of the seminiferous lobules that initiate seasonal recrudescence (Fig. 2). Cyst development begins when a single large cell, the primary spermatogonium, becomes associated with several somatic cells. Mitotic divisions of these primary spermatogonia results in the formation of cysts (enclosed by the seminiferous lobule), containing secondary spermatogonia. Subsequent maturational divisons of these spermatogonia cause an increase in the size of the cysts by the development of spermatocytes that differentiate into spermatids. During spermiogenesis the developing spermatozoa are still enclosed by somatic cells and the integrity of the cysts remains intact. By the time the final stages of spermatozoan maturation are reached, however, the cysts are highly distended. Presumably due to this large increase in size the cysts now rupture, resulting in the spermatozoa (embedded in somatic cells) lying free within the confines of the seminiferous lobule. The release of spermatozoa from the somatic cells, by the act of spermiation, now allows exit of the spermatozoa via the lumen of the lobules into the main collecting duct. It should be emphasized that once the cysts enter into the spermatogenetic process the germ cells present in each of these structures undergo the various developmental changes synchronously in a given seminiferous lobule. Thus, each lobule will, in successive periods, contain in its maturing portion, spermatocytes, spermatids, and finally spermatozoa. The maturing portion will never at any one time contain all, or a combination of, these stages as occurs in the seminiferous tubules of the amniotes.

It has been previously demonstrated that in *Necturus maculosus* the longitudinal wave of spermatogenesis is also reflected in the degree of development of the adjacent interlobular tissue (Humphrey 1921; Pudney et al. 1983). The same relationship also prevails in the testis of

N. lewisi. The interlobular cells associated with the immature region of seminiferous lobules, or those surrounding lobules containing developing germ cells appear, morphologically, to be undifferentiated (see Fig. 2). When, however, the lobules undergo spermiation this, in as yet an

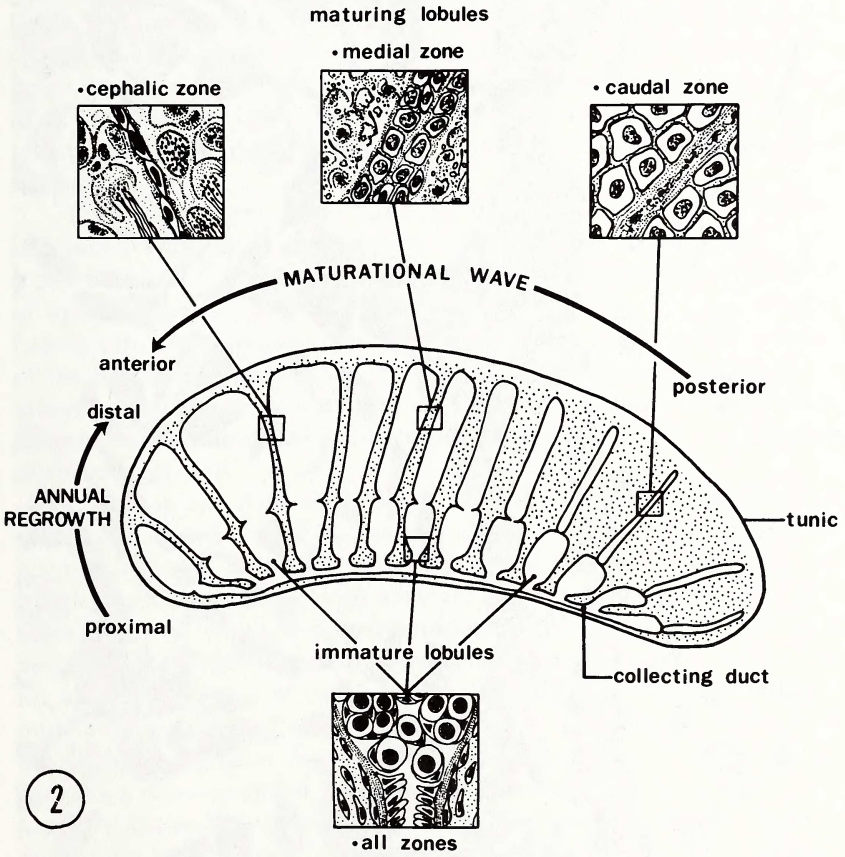

Fig. 2. Diagrammatic representation of *N. maculosus* testis towards the close of spermatogenetic activity. Seminiferous lobules (clear areas) containing the germinal elements empty into the main, central, longitudinal collecting duct. Lobular portions adjacent (proximal) to this duct contain immature germ cells that act as a reservoir for successive bouts of spermatogenetic activity. Mature germ cell cysts are in lobular portions distal to the collecting duct. Lobules are surrounded by an interlobular tissue (grey areas), development of which depends upon the state of differentiation of the lobules. The kinetics of spermatogenesis are indicated by arrows showing the proximal to distal development of germ cells (annual regrowth) and the caudocephalic differentiation of germ cells that occurs in distal portions of the lobules and their associated interlobular tissue during the annual breeding season (maturational wave). From Pudney et al. (1983).

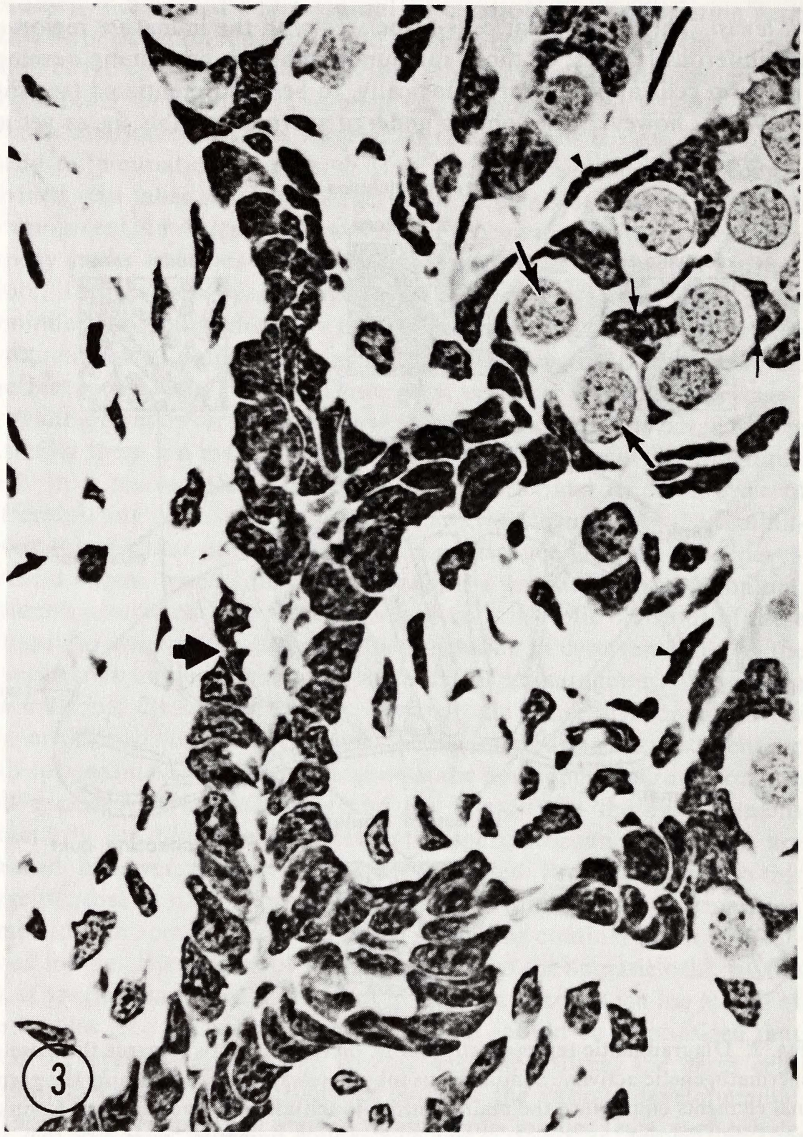

Fig. 3. Section of testis of *N. lewisi* showing seminiferous lobules containing immature germ cell cysts composed of spermatogonia (large arrow) and somatic cells (small arrows). Lobules are connected by short ducts, devoid of germ cells, to the main collecting duct (large arrowhead). Interlobular tissue (small arrowheads) is undeveloped. X 350.

unknown manner, signals the hypertrophy of the interlobular cells surrounding this region. With the subsequent degeneration of the distal lobular portion, following the release of the spermatozoa, the interlobular cells continue to differentiate and eventually form what has been termed the glandular tissue (see Fig. 2). This region was probably first described in urodeles by Perez (1906), but the actual term glandular tissue was first used by Champy (1913). Champy was so struck by the change in color of the testes associated with the formation of the glandular tissue, which is orange/yellow due to the enormous lipid content of the interlobular cells, that he likened its development to the corpus luteum of the mammalian ovary, calling it a "veritable corps jaune testiculaire."

The development of the glandular tissue was described in *Necturus* in a very extensive and comprehensive review by Humphrey (1921). This study established that the glandular tissue was formed at the completion of spermatogenesis and was composed, essentially, of hypertrophied Leydig cells and remnants of degenerating lobules remaining at the close of the breeding season. Since the Leydig cells only differentiate as the lobules undergo spermiation, the evolution of the glandular tissue also proceeds in a caudocephalic direction, following, as it were, in the wake of spermatogenesis.

Observations of *N. lewisi* testis towards the close of the breeding season demonstrate the simultaneous occurrence of discrete regions displaying different stages of germ cell development. Along the entire length of the testes the lobules adjacent to the collecting duct consist of short segments containing spermatogonial cysts (Fig. 3). It should be mentioned that these immature segments are much longer in *N. maculosus* at the same stage of testicular development. This possibly reflects a more extensive period of spermatogenesis in *N. lewisi*, resulting in the depletion of a greater number of spermatogonial cysts, which in turn suggests a more extended breeding period for this species than *N. maculosus*. At the end of the breeding season the immature lobular segments are mostly in a "resting" condition, although a number of mitotic figures were apparent in this region (Fig. 4). Thus, it seems that initial germ cell replenishment can occur, albeit at a slow rate, long before the main wave of spermatogenetic activity takes place. As has also been demonstrated in *N. maculosus* (Pudney et al. 1983), in *N. lewisi* testis the peripheral portions of the lobule undergo a marked longitudinal variation in development. Progressing from the anterior to the posterior end of the testis are seen: 1) highly distended lobules filled with bundles of spermatozoa embedded in somatic cell cytoplasm (Fig. 5); 2) recently emptied lobules containing residual spermatozoa embedded in somatic cell remnants (Fig. 6); 3) lobules in a complete state of collapse and regression (Fig. 7). These zones have no definite boundaries;

they merge gradually, forming a continuum of intermediate stages (see Fig. 5).

In the cephalic zone the lobules contain a patent lumen throughout their length for the egress of released spermatozoa. These spermatozoa enter the Wolffian ducts where they are stored prior to spermatophore formation (Fig. 9). In the central area of the testis the lobules that have undergone spermiation are often still connected to the immaure portions, but at this stage are usually in the process of being pinched off from this part of the lobule (see Fig. 6). Finally, in the caudal zone the degenerating lobular portions become completely separated from the immature regions and reduced in volume owing to the dissolution of the somatic cells by fatty degeneration (see Fig. 7).

The interlobular tissue surrounding these stages of lobular development also display dramatic differences in differentiation. Observed by light microscopy, the interlobular cells associated with immature regions of the lobule resemble fibroblasts in their gross morphology. Observed by electron microscropy, however, they have been identified in *N. maculosus* as poorly differentiated Leydig cells (Callard et al. 1980). In the cephalic zone the distended, sperm-filled lobules occupy almost the entire volume of this region. This made it difficult to locate and identify the interlobular tissue. When this tissue was studied in *N. maculosus* by means of electron microscopy, however, it could be seen to be com-

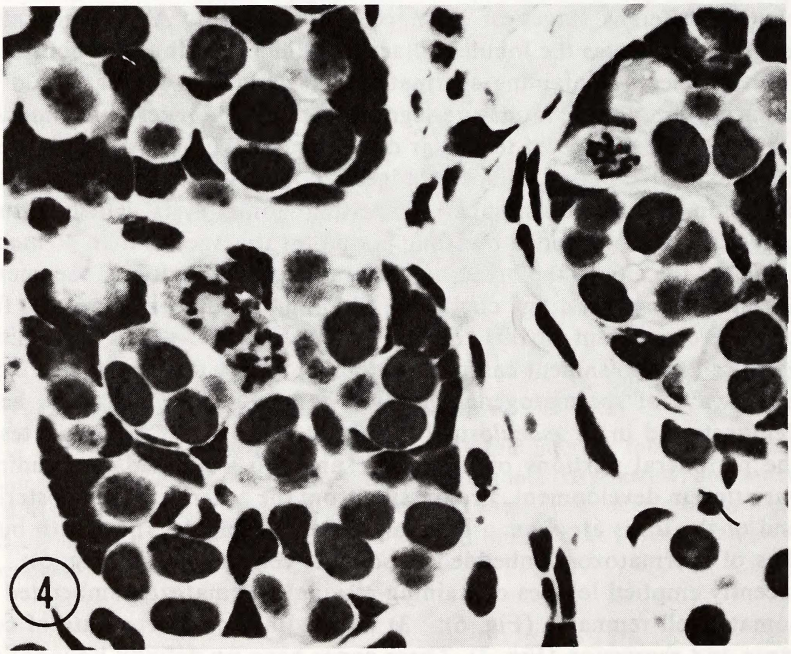

Fig. 4. Mitotic figures occasionally occur in immature cysts. X 357.

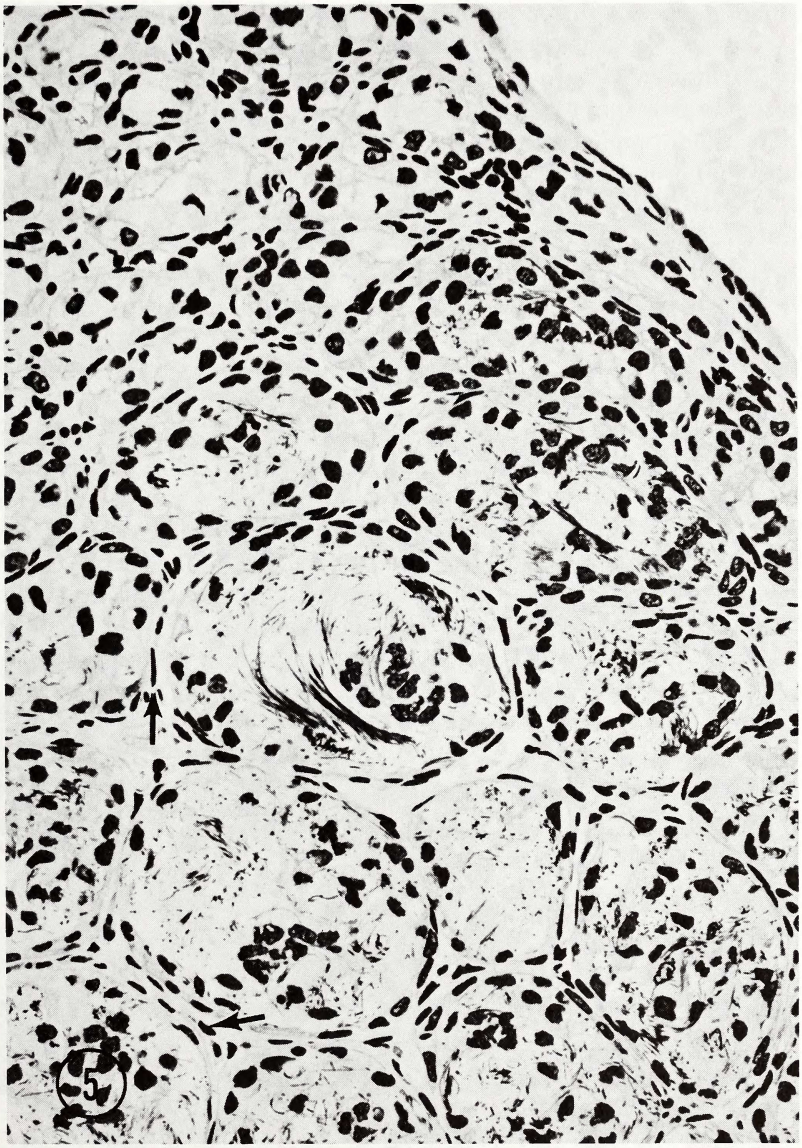

Fig. 5. Cephalic region of testis, containing cysts filled with spermatozoa. Interlobular tissue (arrows) is undeveloped. Note that this region merges with the recently spermiated area at the top. X 121.

posed of immature Leydig cells and myoid cells (Pudney et al. 1983). The Leydig cells often abutted directly against the basal lamina of the seminiferous lobule, but usually were excluded from this area by one or more long cytoplasmic processess developed by myoid cells. Thus, these

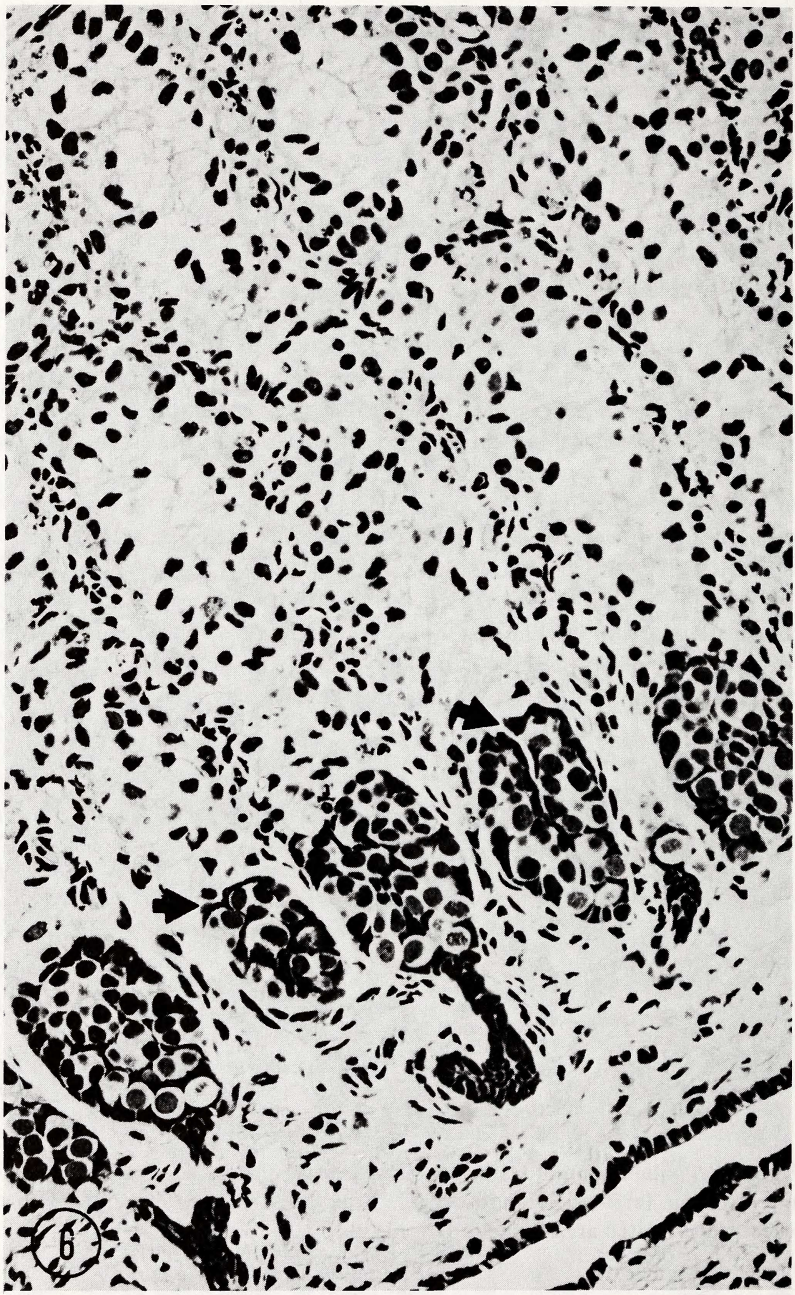

Fig. 6. In the spermiated region of the testis the immature lobular portions have pinched off the mature portions (arrowheads). The mature portions are now degenerating while the interlobular tissue is developing to form the glandular tissue. X 70.

myoid cells resemble, both in appearance and position, those present in the boundary wall of mammalian seminiferous tubules.

Although the intervening stages of lobular development (i.e. spermatocytes, spermatids) are no longer present in the testes of animals at this time, it would appear that development of the interlobular cells is arrested through all stages in which germ cells are present in the lobule. Following spermiation, however, the interlobular cells now become less elongated with rounded nuclei. Electron microscope examination of these cells in *N. maculosus* has demonstrated that they now possess abundant organelles normally associated with active steroidogenesis (Pudney et al. 1983). The regressing portions of the lobules eventually become surrounded by fully differentiated Leydig cells, which in cross section form what has been termed the "epithelioid ring" (see Fig. 9) (Humphrey 1921). This region, which occupies the peripheral part of the testis, between the terminal segments of the immature lobules and the testicular capsule, constitutes the newly formed glandular tissue. In the caudal zone of the testis this finally becomes the glandular tissue proper, which is markedly increased in volume by further hypertrophy of the Leydig cells and complete regression of the lobular remnants. The glandular tissue also becomes highly vascular with groups of Leydig cells closely associated with numerous blood vessels.

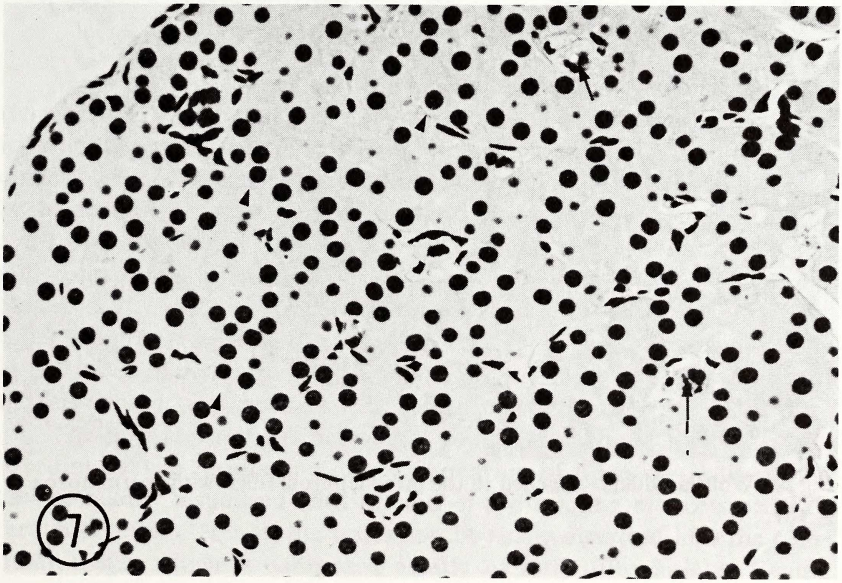

Fig. 7. Glandular tissue proper is composed of hypertrophied Leydig cells (arrowheads) and degenerating remnants of seminiferous lobules (arrows). X 125.

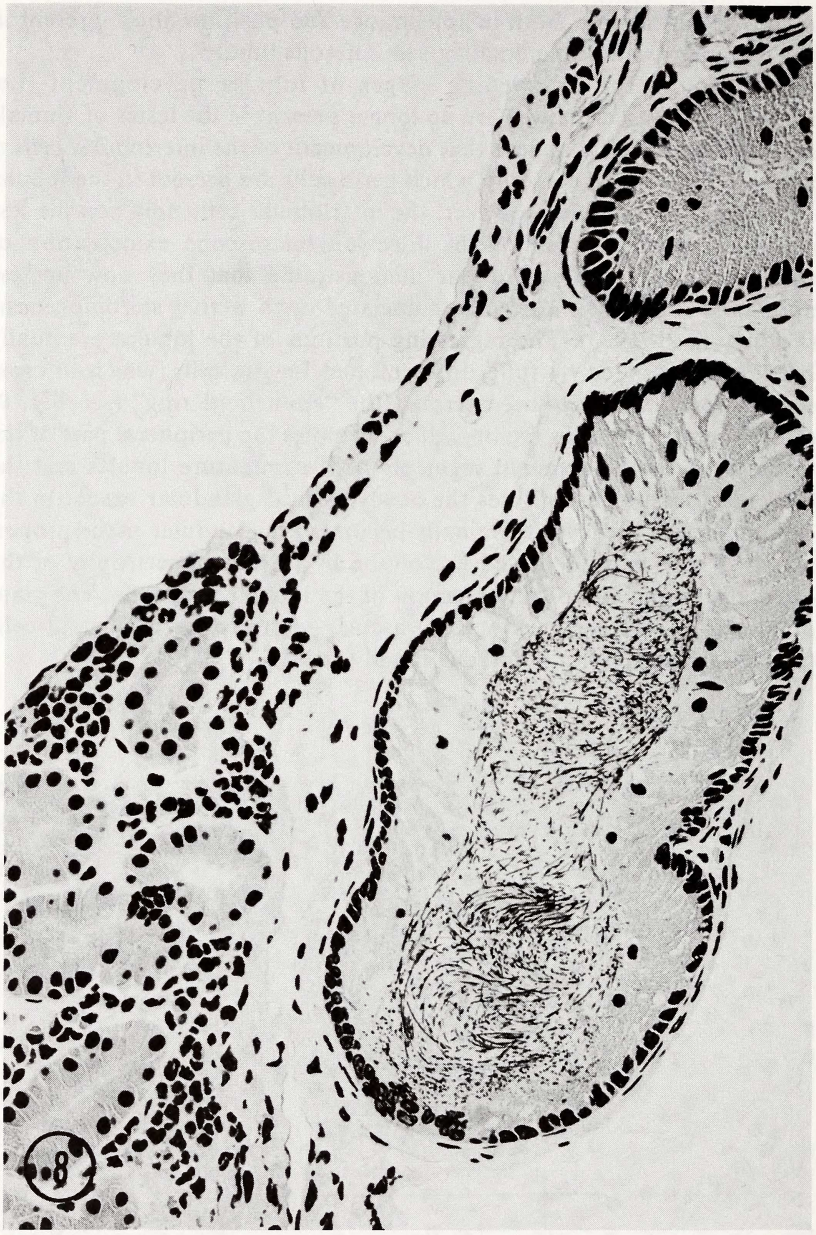

Fig. 8. Wolffian ducts (attached to the mesonephros) filled with spermatozoa. X 101.

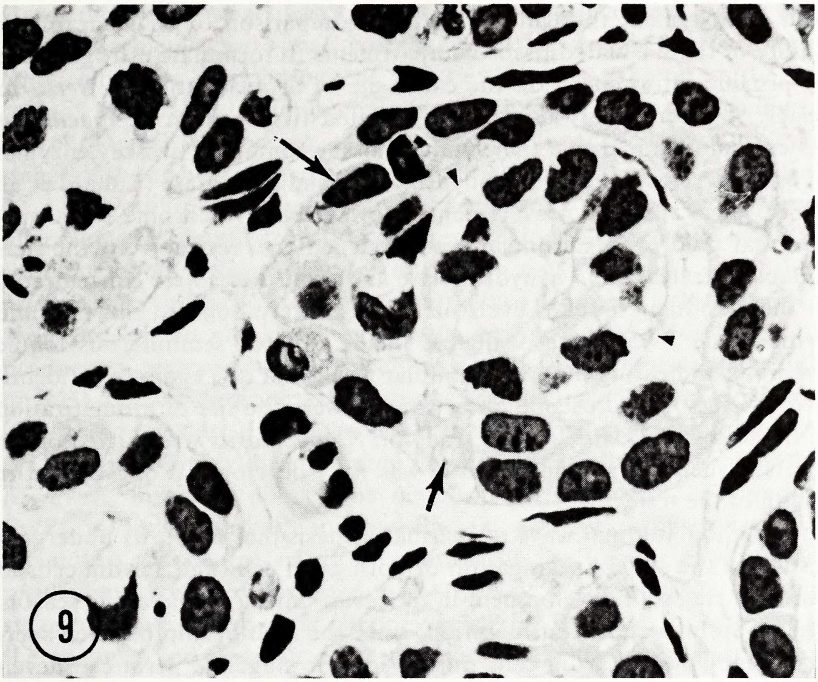

Fig. 9. The "epithelioid ring" of hypertrophied Leydig cells (arrows) surrounding the degenerating lobules (arrowheads). X 330.

STEROID PRODUCTION BY NECTURUS *TESTIS*

We became interested in *Necturus* when it was demonstrated that the testes of *N. maculosus* possessed exceptionally high aromatase activity when homogenates of this tissue were incubated with a steroid precursor [^{3}H]-androstenedione (Callard et al. 1978). Although it has long been recognized that estrogens can be synthesized and secreted by the testis of many species, the exact intratesticualr site of aromatization is still controversial. Thus, both interstitial Leydig cells and intertubular Sertoli cells have been implicated as the source of testicular estrogens. Since the previous study had demonstrated such high aromatase activity in the testes of *Necturus*, we thought they would be convenient animal models to study the specific site of estrogen production.

The formation of the peripheral glandular tissue at the end of the breeding season results in a distinct zonation of *Necturus* testis, which can be readily visualized with the aid of a dissecting microscope (Callard et al. 1980). Hence, the testes can be easily dissected into the glandular tissue, which is composed mostly of fully differentiated Leydig cells and another component comprised of seminiferous lobules containing immature germ cells and their associated somatic cells. This fortui-

tous arrangement thus allows a direct comparison to be made of the ability of these isolated tissue compartments to formulate estrogens and so provide information on the exact site of aromatization in *Necturus* testes. A recent study using these isolated tissues from *N. maculosus* testes demonstrated that the glandular tissue contained two key enzymes in estrogen biosynthesis, 17 α-hydroxylase and aromatase (Callard et al. 1980). Also, spectral measurements showed that cytochrome P-450 species that bind progesterone and androstenedione, respectively, the steroidal substrates for 17 α-hydroxylase and aromatase, were concentrated in the glandular tissue. Levels of both the steroidogenic enzymes and cytochrome P-450 were negligible in the isolated seminiferous lobule fractions, indicating that the glandular tissue and its Leydig cells (identified by electon microscope observations) were the site of aromatization in the testes of *Necturus*. This study on *N. maculosus* was carried out in the fall when the glandular tissue was fully developed along the entire length of the testes.

The longitudinal wave of spermatogenesis that *Necturus* undergoes results in the spatial segregation of both germ cells and Leydig cells at different stages of development in the testes during the breeding season. This is an important consideration, since the relationship between spermatogenesis and Leydig cells during specific stages of germ cell development is very difficult to study in most common laboratory animals. In these species all spermatogenic stages occur simultaneously in the testes from the onset of puberty. Moreover, the seminiferous tubules and interstitial tissues are uniformly distributed and intermingled throughout the entire organ. Therefore, using these species it is technically difficult to obtain precise information on the functional interdependence of the two tissue compartments without disrupting their normal anatomical relationships. These difficulties, however, can be circumvented by studying the testes of *Necturus* with its discrete temporal and spatial segregation of germ cell stages and accompanying interlobular tissue (Pudney at al. 1983).

Figure 2 demonstrates salient morphological features that have been diagrammatically represented for the testis of *N. maculosus* as it appears towards the close of the breeding season (Pudney et al. 1983). At this time of the year essentially the same anatomical arrangment of tissues also occurs in the testes of *N. lewisi*, which were separated transversely into a cephalic region and a caudal region. Both regions contained the immature portions of the seminiferous lobules and associated undifferentiated interlobular tissue. The cephalic region, however, also possessed the maturing lobular portions filled with cysts containing spermatozoa, while the caudal region was composed of the spermiated degenerating lobules plus the hypertrophied Leydig cells forming the glandular tissue. The isolated regions were analyzed for 17 α-hydrox-

ylase and aromatase activity and cytochrome P-450 content. These functional parameters were then correlated, by means of electron microscopy, with the morphology of the Leydig cells present in the two regions. In this way a direct comparison could be made concerning the morphological appearance of Leydig cells, associated with different stages of the spermatogenetic cycle, and their capacity for aromatization.

The results of the assays for 17 α-hydroxylase and aromatase in microsomes prepared from the different regions of *N. lewisi* testes are summarized in Table I. Under our assay conditions, 17 α-hydroxyprogesterone is the sole product synthesized from the substrate, [^{3}H]-progesterone. In all experiments both estrone and estradiol-17 β are products of aromatization using either [^{3}H] 19-Hydroxyandrostenedione or [^{3}H] androstenedione as substrates for this enzyme. Previously we localized these steroidogenic enzymes in fully developed glandular tissue comprised of highly differentiated Leydig cells (Callard et al. 1980). In *N. lewisi*, however, due to the temporal formation of the glandular tissue, we have been able to show that the activity of these enzymes is related to the degree of differentiation of the Leydig cells. Similar results have been demonstrated in the testis of *N. maculosus* at the same stage of the breeding cycle (Pudney et al. 1983). Thus, in both species the enzymes increased in activity progressively from the least mature anterior segment of the testis, containing poorly developed Leydig cells, to the posterior segment possessing the glandular tissue comprised of fully differentiated Leydig cells. Cytochrome P-450, which is the catalytic component of each of the steroidogenic enzymes studied here, was also measured in microsomes prepared from each of the testicular segments. Differences in cytochrome P-450 concentration in these regions was also found to closely reflect the observed changes in activity of these enzymes (Table I).

DISCUSSION

In his study of *N. maculosus*, Humphrey (1921) stated that the glandular tissue developed not only by hypertrophy of the Leydig cells, but also by mitotic activity of these cells. The present study on *N. lewisi* (and also observations on *N. maculosus*) did not demonstrate mitotic figures in the glandular tissue, at any state in its formation. These conflicting observations are difficult to reconcile, unless the mitoses of the differentiating Leydig cells are so transient they can only be detected during extremely short periods of the breeding season, not examined in our investigations.

It would be pertinent to discuss briefly, at this stage, the derivation of the interlobular Leydig cells present in the testis of *N. lewisi*. This is an important issue since it has long been accepted in some anamniote classes, such as teleosts and urodele amphibians, that definitive Leydig

Table 1. Activity of 17 α-hydroxylase and aromatase in microsomes from regions of *Necturus lewisi* testis displaying different degrees of Leydig cell differentiation. Substrates: 19OHA = 19 hydroxyandrostenedione; A = androstenedione; P = progesterone.

Assay date	Testicular segment	Leydig cell development	Aromatase [1,2]		17 α-hydroxylase [1,2]	Cytochrome P450 [1,2,3]
			19OHA	A	P	
2/12/81	Anterior:					
	proximal lobules immature					
	distal lobules distended with sperm	Undifferentiated	33	33	78	0.26
	Posterior:					
	proximal lobules immature	Undifferentiated	202	234	1236	3.20
	distal lobules spermiated	Differentiated				
3/6/81	Anterior:					
	proximal lobules immature					
	distal lobules distended with sperm	Undifferentiated	103	118	—	0.11
	Posterior:					
	proximal lobules immature	Undifferentiated	742	1177	—	0.44
	distal lobules spermiated	Differentiated				

3/30/81	Anterior:					
	proximal lobules immature					
	distal lobules distended with sperm	Undifferentiated	43	50	271	—
	Posterior:					
	proximal lobules immature	Undifferentiated	320	364	1164	1.96
	distal lobules spermiated	Differentiated				

[1] Values represent means of triplicate determinations using microsomes freshly prepared from testicular regions. For experimental details see Callard et al. (1980) and Pudney et al. (1983).

[2] Aromatase and 17 α-hydroxylase — pmol/min/mg protein; P450 concentration — nmol/mg protein.

[3] Cytochrome P450 is the catalytic component of these steroidogenic enzymes, and differences in its concentration closely reflect observed changes in enzyme activity.

cells are not actually present in the interlobular tissue but occur as a component of the boundary wall surrounding the seminiferous lobules. This concept of Leydig cell development was originated by Marshall and Lofts (1956) who suggested, from investigations restricted to light microscopic observations of frozen sections colored with a Sudan dye, that in the teleost testis a lobular boundary cell was the homologue of the Leydig cell present in the testis of these amniotes. This initial observation eventually became expanded to include the urodele amphibia where it has been routinely reported that lobular boundary, perilobular, or pericystic cells were analogous, if not homologous, to Leydig cells of the amniote testis (see review by Lofts 1968; Roosen-Runge 1977; Pilsworth and Setchell 1981). This concept, however, became confused when, from various descriptions of the teleost testis using the more critical resolving power of the electron microscope, it was reported that the lobular boundary cell actually corresponds to the Sertoli cell (see review by Grier 1981). The situation is further complicated in that anamniotes undergo a seasonal cycle of testicular activity, and so, depending upon the stage of spermatogenesis, Leydig cells may or may not appear conspicuous. This becomes an important point if the observations being carried out use the light microscope for examination of testis sections, since the resolution of this instrument often precludes the positive location and identity of Leydig cells. An attempt to clarify the question concerning the presence and identity of Leydig cells in anamniotes has recently been carried out by Grier (1981).

Grier (1981) re-evaluated the literature pertaining to the structure of the teleost testis and concluded that the term lobular boundary cell was no longer tenable as the definition of Leydig cells in these anamniotes. In fact, in this vertebrate group the description of the lobular boundary cells as Leydig cells is erroneous. From its location, and other morphological criteria, the lobular boundary cell actually represents the Sertoli cell of the teleost testis. In view of this, Grier strongly stated that the term lobular boundary cell used to describe Leydig cells in the teleost testis be discontinued. In our studies on *Necturus* testis we have reached a similar conclusion. First, Leydig cells at various stages of differentiation appear to be a constant component of *Necturus* testis throughout the year. Secondly, these Leydig cells are present in the interlobular compartment arising from precursor cells that are a permanent feature of this tissue. Thus, the concept of the lobular boundary cell is also no longer applicable in the species that we have investigated. Furthermore, the presence of myoid cells in the interlobular tissue of *N. maculosus* testis, which often excluded the Leydig cells from direct contact with the seminiferous lobules, is morphologically reminiscent of the anatomical arrangement present in the amniote testis. It would seem, therefore, that the organization of the testis in *Necturus*, and possibly in

all urodele amphibians, is similar to but less complex than that of the amniote testis.

The biochemical parameters measured in *N. lewisi* testis, indicating active steroidogenesis, essentially correspond with the topographical location and morphological differentiation of the Leydig cells. Thus, it would appear that these Leydig cells are responsible for the production of steroids by the testis of *N. lewisi*. Their steroidogenic potential also appears to be related to the cycle of the seminiferous epithelium, since spermiation, followed by regression of the mature lobules, is the event signaling hypertrophy of the surrounding interlobular tissue. It is possible that similar changes also occur in mammals but have never been detected, since not only are different germ cell stages normally found throughout the testis year-round in most common laboratory animals but differentiation is not synchronized in all germ cells of any one testicular region. Recent studies have in fact shown that when Leydig cells from adult rats are separated on density gradients, at least three functionally distinct populations can be identified (O'Shaugnessy et al. 1981). Whether these Leydig cells are randomly distributed throughout the testis of the rat or actually occur in specific relation to certain stages of germ cell development, however, remains to be elucidated. An intriguing question is the mechanism by which the spermiated, degenerating lobules of *Necturus* testis initiate the hypertrophy and differentiation of the Leydig cells associated with these regions. This seems to be a generalized phenomenon, since there are scattered reports in the literature demonstrating, in many mammalian species, that damage to the seminiferous epithelium, either by physical or chemical agents, results in the hypertrophy of Leydig cells adjacent to these lesions (Neaves 1975; Aoki and Fawcett 1978). Furthermore, inhibition of spermatogenesis due to cryptorchidism is similarly associated with an increase in development of Leydig cells as well as an enhancement of androgen secretion (deKretser et al. 1980). As yet, however, further investigations are required to determine the molecular events controlling this interesting relationship between the spermatogenic tissue and Leydig cells.

It has been assumed that, in all species, at least some stages of spermatogenesis are androgen-dependent (Callard, I. P. et al. 1978; Rodriguez-Rigau et al. 1980). In *Necturus* testis, therefore, it seems paradoxical that lobular regions which contain differentiating germ cells are found to be associated with poorly developed Leydig cells possessing low androgen synthesizing abilities. It is possible, however, that these Leydig cells do synthesize low but adequate quantities of androgens that suffice for local spermatogenetic requirements. Among non-mammals it has been commonly demonstrated that spermatogenetic activity and Leydig cell development are often temporally separated during the annual cycle (Lofts and Bern 1972; Callard, I. P. et al. 1978). Where

measurements have been made, it has been found in some species that, although intratesticular androgen levels are high during spermatogenesis, the rise in plasma androgen and the display of breeding behavior corresponds to the time of maximal Leydig cell hypertrophy (Courty and Dufaure 1979). These observations, therefore, lead us to the possibility that the development of a large volume of Leydig tissue, synthesizing high levels of androgen and estrogen, as for example the glandular tissue of *N. lewisi*, may be an adaptation for the secretion of steroids into the circulation (see Pudney et al., 1983). The extensive vascularization of the glandular tissue and the timing of its development following spermiation tends to support this view. The function of these steroids produced by the glandular tissue is unknown, although exceptionally high levels of circulating androgen and estrogen have been measured in *N. maculosus* (Bolaffi and Callard 1979, 1981). Presumably they stimulate and maintain development of the secondary sex organs such as the Wolffian ducts, in which spermatozoa are stored prior to mating, as well as the cloacal glands and other spermatophore producing structural paraphernalia. Furthermore, the role any of these steroids plays in the control of behavioral patterns and nuptial coloration resulting in the successful breeding of *Necturus* can be inferred. Of interest in this respect are reports demonstrating that estrogens in the boar have been shown to be important in conditioning sexual behavior (Joshi and Raeside 1973).

The extraordinarily high rate of estrogen production and its function within the testis of *Necturus* remains, however, obscure. Although some of this estrogen probably affects other body tissues, the presence of receptors in the testis of *Necturus maculosus* (Mak et al. 1983) indicates an action *in situ* as well. It seems reasonable to predict that estrogens may exert a direct inhibitory effect on Leydig cell steroidogenesis and thus signal the demise of the glandular tissue, especially since aromatase activity is maximal towards the end of the breeding cycle. A remarkable parallel occurs in the primate corpus luteum, another highly developed glandular tissue in which degeneration (luteolysis) is initiated by direct application of estrogen (Karsch and Sutton 1976). This, interestingly enough, brings us back to the original observation by Champy (1913) who suggested that the glandular tissue developed by *Necturus* was in fact a "corpus luteum of the testis".

The above account of our studies on *Necturus* testis raises and leaves unanswered many interesting and intriguing questions, both at the morphological and biochemical levels. This is the way of research. In seeking an understanding of nature's mysteries, one is usually left with more imponderables than one started with—which keeps the business of research alive. In the field of male reproduction, despite the efforts of numerous workers past and present, our understanding of the

mechanisms controlling spermatogenesis is still rudimentary. It would appear that in order to approach and elucidate some of these problems more attention should be made in choosing the correct and appropriate animal model. Nature, fortunately, has been very generous to investigators of male reproduction by offering a wide range of animals displaying different reproductive strategies. No one species *per se* is capable of providing all the answers. By judicious selection, however, one can elect to investigate a particular animal because, due to some unique or novel morphological or biochemical parameter, it is more suited for studying one particular aspect of male reproduction. Thus, in *Necturus* testis the development of the glandular tissue plus the formation of high levels of estrogen make this species an excellent animal model for studying the relationship between spermatogenesis and Leydig cells and the role of estrogen in the testis. Both these problems are difficult to approach in the more acceptable laboratory animals, which illustrates and defines the importance of studying the so-called unconventional animal models in order to understand more fully the phenomena associated with male reproduction.

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